

relationship between the host range and virus virulence (Gillard et al., 1986).

## 2.5 Vaccinia virus Ankara

The dermovaccinia strain Ankara (CVA) came onto the market in German in the 1950s as a pox vaccine, and was attenuated in more than 500 passages on embryonic chicken fibroblast (HEF) cell cultures. Beginning with the 516<sup>th</sup> passage on HEF cells, the virus strain was given the name Modified Vaccinia Virus Ankara (MVA) (Mayr et al., 1975).

The MVA virus can be clearly differentiated from other species of orthopox viruses by its stable biological markers. Especially noticeable is its reduced virulence for the chicken embryo, for research animals and for human beings. When the vaccinia virus strain MVA – in contrast to other vaccinia viruses – was tested in a large number of rodents and apes, no amplification of virulence was observed, even in immunosuppressed animals (Mayr et al., 1978). The MVA virus proved to be suitable for pox vaccination in human beings after more than 120,000 people received initial vaccinations with the virus without complications (Stickl et al., 1974; Mayr and Danner, 1979).

In contrast to the original strain, CVA, the MVA virus strain possesses a narrowed host range in cell cultures. The abortive replication of the MVA virus in human cell cultures was used to assess the safety of the virus for first-time vaccines (Mayr et al., 1978).

In experiments involving restriction enzymes, Altenburger et al. (1989) examined the genome of the vaccinia virus strains MVA and WR. In the genome of the MVA virus strain, they found, in addition to the attenuation of the HindIII fragments B and C, a deletion of 2.5 Kbp in the region of the M/M fragments, which results in the loss of a HindIII interface. A sequence comparison of this genome region in vaccinia viruses MVA and WR revealed, in the genome of the MVA, the loss of the entire gene of a 55 KD polypeptide with as yet unknown function. At the same time, more than two-thirds of the host range gene were lost. While Gillard et al. (1986) determined the gene product as a 29 KD polypeptide, Altenburger et al. (1989) calculated a 32 KD protein. In addition, four other deletions, though very small (1, 14, 16

and 17 bp), and several nucleotide transitions were found in the genome of MVA. The small 14 bp deletion lies in the promoter region of the host range gene. Conspicuously, the MBA virus strain was able to replicate further on the human cell line 143 BTK, whereas this cell line was no longer permissive for a virus mutant absent in the entire host range (Drillien et al., 1981).

A direct comparison of virus strains CVA and MVA is intended to highlight the changes in the genome of two phenotypically different but closely related virus strains that have occurred during the passage time. Of special interest is whether the change in the host range gene region can be viewed as the sole cause of reduced virulence, or whether other changes also play a role.

### 3. Material and methods

#### 3.1 Cell cultures

For virus replication and/or to determine the host range, the cell cultures and cell lines listed in Table 1 were used and cultivated in accordance with standard methods (Mayr et al., 1974). The culture media used were Minimum Essential Medium, with the addition of Earle's salts (EMEM), Medium TC 199 or RPMI 1640 Medium, to which either fetal calve serum (FKS) or Basal Medium Supplement (BMS) was added (all media and salts were from the Biochrom Company, Berlin).

Primary embryonic chicken fibroblast cell cultures (HEF) and bovine embryonic lung cell cultures (BEL) were produced from chicken eggs (Heinrichsruh Farm, Langenbach) incubated for 10 days and from 3-4 month old cow fetuses (Munich Slaughterhouse), using the method of fractionated trypsinization described by Mayr et al. (1974).

Table 1: Composition of the cell cultures used

Designation and source		Passage (P)
HEF	Embryonic chicken fibroblasts	prim./sec.
BEL	Bovine embryonic lungs	prim./sec.
MA 104	Monkey kidney cells	34.
VERO	Monkey kidney cells	132.
E-DERM	Equine dermal cells	28.
MDBK	Bovine kidney cells	114.
MDCK	Canine kidney cells	217.
RK 13	Rabbit kidney cells	96.
DBT	Mouse tumor cells	6.
LSCC-H-32	Chicken fibroblast cell line	132.
HELA	Human epithelial carcinoma	13.
HEL	Human embryonic lungs	13.
HRT 18	Human rectal tumor cell line	30.
HEP 2	Human epitheloid carcinoma	6.

#### 3.2 Virus strains

Of vaccinia virus Ankara (Herrlich and Mayr, 1957; Mayr et al., 1975), the 2<sup>nd</sup>, the 382<sup>nd</sup> and the 574<sup>th</sup> passage were examined on embryonic chicken fibroblasts (HEF) and the 486<sup>th</sup> passage on embryonic pig kidney cells (ENS).

The WHO vaccinia virus reference strain Elstree (Lister Institute) served as the control.

At the beginning of the experiments, all virus strains were cloned three times, using the plaque method (Mayr et al., 1974), on HEF secondary cultures and/or on the cell line MA 104 (Elstree). In the following, the plaque-cleaned 2<sup>nd</sup> HEF passage of the vaccinia strain Ankara is referred to as CVA 2, the 382<sup>nd</sup> HEF passage as CVA 382, the 574<sup>th</sup> HEF passage as MVA 574, and the 486<sup>th</sup> ENS passage as CVA ens.

The 5.2 Kbp EcoRI fragment of the DNA of CVA 2 was inserted by transfection into the genome of MVA 574 and CVA 382 (see section 3.3). This fragment overlaps a deletion described for MVA 574 in this region, and contains the complete vaccinia virus "host range" gene (Altenburger, 1989). The constructs created through transfection are abbreviated in the following as #MVA and #CVA 382.

### 3.3 Marker rescue experiments

#### Material:

Calcium chloride gradient cleaned plasmid DNA of the clone pT% 5.2 (contains the 5.2 Kbp EcoRI fragment of CVA 2; for production and cleaning, see Mayr et al., in preparation).

2 M CaCl<sub>2</sub>, sterile-filtered

Hepes saline buffer (HEBS), 5x parent solution:

0.7 M NaCl, 25 mM KCl, 3 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM dextrose, 110 mM Hepes, pH value adjusted with NaOH to exactly 7.05

Herring sperm DNA: 10 mg/ml

The cloned 5.2 Kbp EcoRI fragment was inserted into the cells as calcium phosphate precipitate, using the principle of Graham and Van der Eb (1973) (transfection). To this end, 1 µg pT% 5.2 plasmid DNA and 9 µg herring sperm DNA were added to 1 ml of HEBS buffer (DNA concentration of 10 µg/ml), to which 62 µl CaCl<sub>2</sub> were added drop by drop, and the mixture was incubated at room temperature for 30 min., until a fine precipitate had formed. As negative control, the same method was used to precipitate herring sperm DNA only or plasma DNA only, without the insert.

We chose growth on the E-DERM cell line as a screening system for a functioning host range gene region.

The highly passaged virus strains CVA 382 and MVA 574 cannot replicate on these cells, due to their limited host range. Subconfluent equine dermal cells were infected with 0.05 plaque-forming units (PBE) per cell of the vaccinia virus strains MVA 574 and CVA 382. Following one hour of adsorption at room temperature, the inoculum was removed, and 1 ml of plasmid/herring sperm DNA precipitate per cell culture bottle was poured directly on the cell lawn and incubated for 30 min. at 37°C. One and a half hours after infection, EMEM with 5% FKS was added, and the medium was replaced (EME, 5% PKS) after three hours. The transfixed cell cultures were generally incubated for 2 to 7 days at 37°C, then freeze-thawed and additionally passaged. Only virus-infected cells were used as the control, and were further observed in five blind passages. Following the occurrence of a cytopathic effect, the virus material was harvested and plaque-cleaned in the same cell system. The insertion of the plasmid insert into the genome was monitored by means of restriction enzyme analysis.

### 3.4 Virus replication

#### 3.4.1 Initial material

Densely grown, primary HEF cultures were infected with 1-2 PBE per cell of vaccinia strains CVA 2, CVA 382, MVA 574 and CVA ens, as well as with the constructs #MVA and #CVA. The virus harvest occurred after 2 to 4 days, at a cytopathic effect (CPE) of 90-100%, by means of double freeze-thawing, in order to release the largely cell-bound virions. To remove larger cell fragments, the virus material was centrifuged at a low speed and, following titration of infectiousness, aliquoted and stored at -70°C.

This virus material, referred to in the following as initial material, was used in all subsequent experiments.

#### 3.4.2 Determination of the host range

The cell lines and/or cell cultures listed in section 3.1 were cultured in plastic cell culture flasks (25 cm<sup>3</sup>, Becton Dickinson Co., Oxnard, Switzerland) and, following removal of the medium by suction, inoculated with CVA 2, MVA 574 and #MVA virus initial material. The inoculation dose was 0.05 PBE per cell. The inoculum was adsorbed for 45 min. at 37°C and

then suctioned off. Following a washing step, 5 ml of medium with 3% BMS/FKS were added to each flask and incubated at 37°C.

After 0, 24 and 72 hours per inf., the cytopathic effect was evaluated, the corresponding cultures were freeze-thawed twice, briefly exposed to ultrasonic waves, and stored at -70°C until titration.

#### 3.4.3 Virus titration

The virus material was titrated on secondary HEF cultures in 96-hold microtiter plates (Becton Dickinson Co., Oxnard, Switzerland). 100 µl of virus dilution in EMEM ( $10^{-1}$  –  $10^{-9}$ ) and 100 µl of cell suspension (500,000 cells/ml) were pipetted into every 8 wells in the flat-base plate. Following brief shaking, the plates were incubated for 6 days at 37°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>). The final reading was done on the basis of the cytopathic effect. The titer of the virus material was indicated, in accordance with Spearman and Kaerber (lit. in Mayr et al., 1974), as a culture-infectious does 50 per ml (KID<sub>50</sub>/ml).

#### 3.5 Hemagglutination (HA)

In the hemagglutination test, CVA 2, CVA 382, MVA 574, CVA ens, #MVA, #CVA 382 and, as control strain, vaccinia virus Elstree were tested.

To this end, the initial material was diluted on U-base microtiter plates (Greiner Co., Nürtingen) in log<sub>2</sub> steps in NaCl-phosphate buffer. Then 0.05 ml of a 0.5% chicken erythrocyte suspension (white Leghorn) in NaCl-phosphate buffer was added to each well, the plates subsequently shaken, and incubated at room temperature. An HA-positive vaccinia virus strain (Elstree) and a non-virus-infected cell culture preparation served as controls. After approx. 2 hours, the highest dilution at which complete hemagglutination still occurred was defined as the HA titer.

#### 3.6 Behavior in the incubator egg

All incubator eggs were obtained from white Leghorn hens from the same poultry farm. Following 10 days of pre-incubation at 37°C, the chorioallantois membrane (CAM) was infected

by means of tray windowing and reduction in the CAM. Every three eggs were inoculated with  $10^1$ ,  $10^2$ ,  $10^3$  and  $10^4$  KID<sub>50</sub>/0.1 ml. The subsequent incubation took place at 36°C. On the fourth day post-inf., the character of the primary foci was determined on the CAM, the death rate determined and the generalization tendency evaluated.

### 3.7 Behavior in the white mouse

#### Test animals:

Female mice from the NMRI breed strain, aged 6 weeks, and 2-3 day old NMRI baby mice of both genders were used for the experiments.

#### Virus material:

For infection, the initial material of vaccinia virus strains CVA 2, MVA 574 and #MVA were adjusted to a titer of  $3 \times 10^{[illegible]}$  KID<sub>50</sub>/ml.

#### Experiment completion:

Each of the adult mice was infected with 1 ml of virus suspension intra-peritoneally (i.p.) or with 0.05 ml of virus suspension intracerebrally (i.c.). Baby mice could be administered 0.1 ml i.p. or 0.02 ml i.c. For the vaccinia virus strains CVA 2 and MVA 574, a group of 10 adult mice and a litter of baby mice was used for each application type. 30 adult animals and 3 litters of baby mice each were infected with the vaccinia virus construct #MVA. The intracerebral application was done under ether anesthesia.

### 3.8 Virus cleaning

#### Material:

TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)  
20%, 40% and 60% (w/w) sucrose in TE buffer  
Fluorocarbon (Frigen 113 TR-T, Hoechst Co., Frankfurt)

The cleaning of virus infected cell culture material (infected with 5-10 PBE/cell) was done in analogy to the method described by Joklik (1962). To this end, the freeze-thawed cell culture material was pelleted for 90 min. at 23,000 g in a refrigeration centrifuge (Measuring and Scientific Equipment Ltd., London),

the residue discarded, and the virus-containing sediment dissolved into TE buffer. This suspension was purged of cellular material by means of fractionated fluorocarbon treatment (Kern, 1987). Released virus particles were subsequently pelleted through a 40% sucrose cushion (10 ml) in the ultra-centrifuge (SW 28 Rotor, 25,000 RPM, 60 min., Beckman Co., Munich). Following re-suspension of the virus-containing pellet in TE buffer, further cleaning was done across a linear 20-60% sucrose gradient (SW 40 Rotor, 15,000 RPM, 90 min., Beckman Co.). The virus material, appearing as a visible band, could be removed by aspiration and, following quadruple dilution with TE buffer, de-sugared (SW 40 Rotor, 25,000 RPM, 45 min.).

As an alternative to cleaning across sucrose gradients, the pelleted virus material was centrifuged twice through a 40% sucrose solution (4 ml) (SW 60 Ti Rotor, 25,000 RPM, 60 min., Beckman Co.). The cleaned virus material, dissolved in a small amount of TE buffer, was stored at -70°C.

To verify cleaning efficiency, the virus material was examined under an electron microscope following each centrifugation. To this end, carrier nets prepared with the applicable virus material, following negative contrasting with 2% phosphotungstic acid, were examined under a transmission electron microscope (M 10, Zeiss Co.).

The protein content of the cleaned virus suspension was determined in a modified Micro-Lowry test (Protein Assay Kit, Sigma Co., Munich).

### 3.9 Identification of viral proteins

#### 3.9.1 Polyacrylamide gel electrophoresis (PAGE)

The separation of the virus proteins was done by discontinuous electrophoresis, in accordance with Laemmli (1970), in a 12% acryl-bisacrylamide gel (30:0.8 w/v) under reducing (by addition of  $\beta$ -mercaptoethanol) conditions. For separation, 10  $\mu$ g of cleaned virus material was used in each case. The electrophoresis time was 16 hours at 50 V in an electrophoresis device in accordance with Studier (1973). A portion of the PAGE gel was

dyed silver to depict the protein bands (Merril et al., 1981), while the non-dyed portion of the gel was blotted.

### 3.9.2 Immunoblot (Western Blot)

Material:

Nitrocellulose, 0.20  $\mu$ m pore size (Schleicher & Schüll Co., Dassel)  
HRP Color Development Reagent (Biorad Co., Munich)

Hyperimmune serums:

(graciously provided by Prof. Dr. H. Mahnel and Dr. C.-P. Czerny)

- Anti-MVA rabbit serum
- Anti-Elstree sheep or rabbit serum
- Monoclonal mouse antibody (MAK): 5B4

For protein identification, the hyperimmune serums were used in an application dilution of 1:100, and the MAK 5B4 at 1:150.

Using the immunoelectroblot method according to Towbin et al. (1979), the virus proteins separated in the PAGE gel were transferred onto nitrocellulose (Transfer Electroblothing chamber, Pharmacia LKB Co., Freiburg, 120-180 min. at 0.7-0.9 A). It was subsequently possible to identify the immunologically reactive proteins with hyperimmune serums and/or MAK 5B4 and the ensuing immune coloration by means of an enzyme-substrate reaction.

### 3.9.3 Reaction with monoclonal antibodies (MAK)

The virus strains CVA 2, CVA 382, MVA 574, #MVA and #CVA 382 were examined in a diagnostic ELISA for differentiation of orthopox viruses (Johann et al., in preparation). In addition to differentiating the individual orthopox species, this ELISA also differentiates within the vaccinia virus species. In this process, nine different MAK bound to the microtiter plate serve as antigen catchers. The identification of bound pox virus antigens (initial material) is done through polyclonal hyperimmune serums with anti-species enzyme conjugates and substrate reaction.

## 3.10 Examination of the virus genome

### 3.10.1 Isolation of viral DNA

Material:

Eppendorf reaction flasks 3810 (1.5 ml), siliconized

TEN buffer (10 x TEN): 100 mM Tris, 10 mM EDTA, 1 M NaCl, pH 7.4, autoclaved

Proteinase K (Sigma Co., Munich): 1 mg/ml (w/v) in 1.5 mM CaCl<sub>2</sub>

SDS, DNase free (Sigma Co., Munich): 20% (w/v)

Phenol, neutralized (produced in accordance with Maniatis et al., 1982)

IAC (1 part isoamyl alcohol and 24 parts chloroform)

3 M sodium acetate pH 4.8

Ethanol, absolute or 70% (stored at -20°C)

300 µl of cleaned virus suspension were combined with TEN buffer (final concentration 10 mM Tris, 1 mM EDTA, 100 mM NaCl), proteinase K (final concentration 100 µg/ml) and SDS (final concentration 1%), mixed and incubated for 1 to 3 hours at 56°C. Through careful end-to-end rotation and double phenol and IAC extraction, the viral DNA was purged of protein remnants. The precipitation of the released DNA was done by addition of 1/10 volume sodium acetate and double volume of absolute, -20°C ethanol, either overnight (at -20°C) or for 30 min. (at -70°C). The DNA was pelleted by ten-minute centrifugation at 4°C at 10,000 RPM (MK 202 refrigeration centrifuge, Sigma Co.). Following double washing with 70% cold ethanol, the DNA pellet was briefly vacuum-dried and re-suspended in a small amount of TE buffer. The DNA concentration was determined by measurement in a photometer (1 OD at 260 nm = 47.5 µg double-stranded DNA/ml) and adjusted with TE buffer to 0.5 µg/µl.

### 3.10.2 Gel electrophoresis

#### 3.10.2.1 Agarose gel electrophoresis

Material:

Horizontal submarine electrophoresis chamber (Pharmacia LKB Co., Freiburg)

UV transilluminator 302 nm

Immediate image camera with red filter and instant pack film type 667 or type 665 with negative (Polaroid Co., USA)

Electrophoresis buffer, concentrated 50x (50xEP): 2 M Tris, 0.05 M EDTA, 0.25 M sodium acetate, pH 7.8

0.5-1.5% agarose Seakem<sup>[illegible]</sup> (Biozym Co., Hameln) in 1xEP

Ethidium bromide (Sigma Co., Munich) parent solution: 10 mg/ml, application dilution 1:10,000 in 1xEP

Stop buffer (10xBSE): 0.25% bromophenol blue, 5% SDS, 50% sucrose, 0.5 M EDTA in distilled water

Restriction endonucleases with specific react-buffer solutions (Boehringer Co., Mannheim): HindIII, XhoI, SmaI, EcoRI, ca. 10 units/ $\mu$ l

The viral DNA was incubated, according to the manufacturer's instructions, for 1-3 hours at 37°C with the applicable enzyme. The addition of stop buffer (1/10 vol.) and heating to 65°C for 10 min., followed by immediate cooling on ice, terminated the enzyme reaction. The separation of the DNA fragments was done in agarose gel at 2-3 volts/cm gel for 20 hours. KB ladders (BRL Co., Eggenstein) and lambda DNA cut with HindIII were used as length standards. For later evaluation purposes, the gel was photographed at 302 nm UV light.

### 3.10.2.2 Polyacrylamide gel electrophoresis (PAGE)

#### Material:

Electrophoresis equipment according to Studier (1973)

Electrophoresis buffer: 0.025 M Tris  
0.192 M glycine  
0.1 % SDS, pH 8.3

The separation of the DNA fragments obtained by digestion with restriction enzymes was done, in accordance with the method of Laemmli (1970), in a 7.5% acryl-bisacrylamide gel (30:0.8 w/v in distilled water) overnight at 50 volts. Following depiction of the DNA bands by silver coloration (Merill et al., 1981), the gel was photographed, transferred to filter paper and dried.

### 3.10.3 Identification of the genome terminal fragments

By the addition of formamide (Fluka Co., Buchs, Switzerland, stored at -20°C) in a final concentration of 60% (v/v) relative to the reaction mixture, the virus DNA, cut with restriction enzymes (see section 3.10.2.1), was denatured for 6 min.

at 60°C, and was subsequently immediately cooled on ice (cross link preparation). In this process, only the covalently bound (cross-linked) individual strands of the DNA terminal fragments quickly become realigned adjacent to one another ("snap-back" mechanism), while all other fragments remain single-stranded. For this reason, the terminal fragments can be depicted in agarose gel following electrophoresis and identified in comparison to the DNA band patterns of the respective enzyme (Mackett and Archard, 1979).

#### 3.10.4 Determination of the size of the virus genome

To determine the total genome length, the molecular weights of the viral DNA fragments obtained by restriction digestion were summed. Fragments with high molecular weight (> 20 Kbp) were isolated from the agarose gel (see section 3.10.5.4) and, for a more detailed analysis, sub-digested again with a second restriction enzyme. With the aid of the molecular weight standards KB ladder and lambda HindIII, it was then possible to determine the fragment sizes by adding together the sub-fragments.

The exact determination of the size of the DNA fragments was done with the aid of a computer program for sequence analysis (Microgenie™, Beckman Co.) on a sound digitizer (Beckman Co.).

#### 3.10.5 Isolation of DNA fragments

##### 3.10.5.1 Freeze squeeze method

To release the DNA, DNA fragments cut out from the agarose gel were each freeze-thawed twice for 30 min. -70°C/room temperature. Following brief centrifugation through a filter made of siliconized glass wool, the DNA solution, purged of gel residues, was extracted with phenol and IAC. The DNA was precipitated with ethanol, pelleted, vacuum-dried and dissolved into 5-10 µl TE buffer (see section 3.10.1).

##### 3.10.5.2 DEAE membrane method

Material:

DEAE membrane Na-45 (Schleicher & Schuell Co., Dassel)

Wash buffer: 0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8.0 autoclaved

Elution buffer: 1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8.0 autoclaved

This method was then used for fragments with low molecular weight ( $< 7$  Kbp). Following separation of the DNA bands in agarose gel, one strip each of Na-45 membrane was placed into the gel both above and below the DNA fragment to be isolated (Winberg and Hammarckjold, 1979). This was done under visual inspection (UV light, 366 nm). The electrophoresis was continued until the DNA band was completely bound on the lower DEAE membrane (visual inspection in UV light). Agarose residues were subsequently rinsed from the DEAE strip with the bound DNA using cold wash buffer, and the strip was then transferred to an Eppendorf reaction flask containing 300  $\mu$ l of elution buffer. Following 45 min. of incubation at 60°C in an agitated water bath, the DNA-containing fluid was obtained, the ethidium bromide extracted with triple volume water-saturated n-butanol, and the DNA precipitated with ethanol (section 3.10.1). The vacuum-dried pellets were dissolved in 5  $\mu$ l of TE buffer. DNA obtained in this fashion could be used for labeling by means of Nick Translation (section 3.11.2.1) or Random Oligo Labeling (section 3.11.2.2).

### 3.10.5.3 Electrophoresis in LMT agarose

This method is based on the method described by Maniatis et al. (1982).

Material:

Low Melting Temperature (LMT) agarose (BRL Col., Eggenstein)

2-4  $\mu$ g of virus DNA was cut with restriction endonucleases and electrophoretically separated in a preparative agarose gel. The desired DNA band was cut out as a small block of agarose and transferred to the prepared slit of a 1% LMT agarose gel. The gel slit was filled with 56°C warm agarose. Then the electrophoresis was continued until the DNA had completely drifted into the LMT agarose. The DNA fragment was then once again cut from the gel as accurately as possible. 3  $\mu$ l of twice-distilled water were

added for each mg of agarose, the mixture boiled for 7 min. at 100°C, and then equilibrated for 10 min. at 37°C. The DNA-agarose mix was then aliquoted in portions of 20-50 ng DNA each and stored at -20°C. If necessary, the DNA samples obtained in this manner could be directly labeled by means of Random Oligo Labeling (see section 3.11.2.2) and then used for hybridization.

#### 3.10.5.4 Electrophoretic elution

Material:

LKB 2014 Extraphor Electrophoretic Concentrator (LKB Pharmacia Co., Freiburg)

The buffer chambers of the elution device were filled with 1xEP, and 3 M of sodium acetate was carefully under-layered in the V-shaped connecting channels.

The DNA fragments, each containing a minimum of 200 ng DNA, were cut out of the preparative agarose gel in the form of agarose blocks and, following comminution, placed into the sample openings of the Extraphor chamber. Following electrophoresis at 80 V for 20-60 min., the DNA sample had drifted into the high-salt trap. The sodium acetate-DNA mixture, removed with a cannula, was diluted 1:10 with twice-distilled water and the eluted DNA was precipitated with 2.5x volume absolute ethanol. The DNA pellet obtained was washed two times in 70% ethanol, vacuum-dried and dissolved in TE buffer.

DNA fragments with high molecular weight were isolated using this method. The DNA obtained could then be re-cut once again with restriction enzymes and depicted in agarose gel.

### 3.11 Test for viral DNA

#### 3.11.1 Southern Transfer

Depending on the carrier matrix used, the method according to Southern (1975) was applied using either a unidirectional blot procedure or as a double-sandwich blot.

Material:

Nitrocellulose membrane BA 45, 0.45 µm (Schleicher & Schuell Co., Dassel)

Hybond-N<sup>+</sup> Transfer Membrane (Amersham Co., Braunschweig)

3 MM chromatography paper (Whatman Co., Maidstone, GB)

Denaturing buffer 1: 0.25 N HCl

Denaturing buffer 2: 0.5 N NaOH / 0.9 M NaCl

Neutralization buffer: 1 M Tris-HCl / 0.9 M NaCl, pH 7.4

SSC buffer: concentrated 20 times (20xSSC): 3 M NaCl, 0.3 M sodium citrate (pH 7.0)

In an agarose gel, 400 ng per slot of DNA cut with restriction enzymes were used and separated. Following photographing, the agarose gel was decolorized for 30 min. in distilled water, and the DNA was denatured for 2x10 min. in HCl buffer and 2x15 min. in NaOH/NaCl buffer.

For transfer to the nitrocellulose filter, the gel was neutralized for 60 min. in Tris-HCl/NaCl, then equilibrated for 20 min. in 6 x SSC and blotted using the double-sandwich method.

Blot structure  
(from top to bottom):

- Glass plate
- Cellulose stack
- 3 Whatman filters in gel size
- Nitrocellulose filter
- Gel
- Nitrocellulose filter
- 3 Whatman filters in gel size
- Cellulose stack
- Glass plate

Neutralization was not necessary for transfer to loaded carrier membranes (Hybond-N<sup>+</sup> Transfer Membrane). The diluted NaOH denaturing buffer, in a final concentration of 0.25 N NaOH, 0.9 M NaCl, served as transfer buffer for the unidirectional capillary blot. The transfer membrane and the Whatman filter were softened in the transfer buffer.

Blot structure for the unidirectional capillary blot  
(from top to bottom):

- Buffer tank with transfer buffer
- Glass plate
- Whatman buffer bridge
- 2 Whatman filters in gel size
- Gel
- Transfer membrane
- 3 Whatman filters in gel size
- Cellulose stack
- Glass plate

The duration of transfer with the double-sandwich blot was 2-4 hours, and 12-16 hours with the unidirectional capillary blot. The DNA was subsequently fixed on the carrier membrane by 2 hours of incubation at 80°C in a vacuum drying cabinet.

### 3.11.2 Labeling of viral DNA

#### 3.11.2.1 Labeling by means of Nick Translation

Reaction principle: A controlled DNase digestion leads to individual strand fracture ("nick") in the double-stranded DNA. As a result of the endonuclease activity of the DNA polymerase (Kornberg enzyme), this individual strand fracture is enlarged and simultaneously closed again, with the inclusion of biotin-11-UTP, as a result of the polymerase activity of the enzyme.

#### Material:

Nick Translation Reagent kit (BRL Co., Eggenstein)

SDS, DNase free, 5% (w/v), sterile-filtered

Sephadex mini-column: Sephadex G50 coarse (Pharmacia Co., Freiburg) in 50 mM Tris-HCl (pH 7.5) with 0.1% SDS was filled into a 1 ml disposable syringe closed with siliconized glass wool and centrifuged at 300 x g for exactly 3 min.

The reaction batch was prepared on ice:

x µl	DNA sample
5 µl	Nucleotide solution (dATP, dCTP, dGTP)
2.5 µl	Biotin-11-UTP
ad 45 µl	Twice-distilled water
	Mix briefly
5 µl	DNase-polymerase mix
90 min.	incubation at 15°C
5 µl	Stop buffer (300 mM EDTA)
1.25 µl	5% SDS

To cleanse the labeled DNA of free nucleotides, the batch was applied to the Sephadex mini-column and centrifuged as described above. The labeled DNA, captured in an Eppendorf flask, could be used for hybridizations immediately following denaturing (10 min. 95°C).

### 3.11.2.2 Labeling by means of Oligo Labeling

Reaction principle: DNA is labeled by inclusion of digoxigenin-dUTP, with oligonucleotides serving as starter molecules for the synthesis reaction ("random primed"). This method can be used directly for labeling DNA in LMT agarose.

#### Material:

DNA Labeling and Detection Kit Nonradioactive (Boehringer Co., Mannheim)

Stop buffer: 250 mM EDTA in twice-distilled water, autoclaved

t-RNA (Boehringer Co., Mannheim): 0.25 mg/ml in TE buffer

The DNA sample was denatured for 10 min. at 95°C, then immediately placed onto ice. Following boil up, DNA samples in LMT agarose were incubated for 5 min. at 37°C and then briefly mixed.

The reaction batch was prepared on ice, but at room temperature for labeling of DNA in LMT agarose:

x µl	DNA sample (max. 10 µl)
2 µl	Hexanucleotide mixture
2 µl	dNPT labeling mixture
	(dATP, dCTP, dGTP, dTTP, Dig-dUTP)
ad 19 µl	Twice-distilled water
	Mix briefly
1 µl	Klenow enzyme
	Incubation for LMT samples 12-20 hours at RT
	otherwise 60 min. at 37°C
2 µl	Stop buffer
1 µl	t-RNA

Non-included nucleotides were removed by centrifugation over a Sephadex G50 mini-column (see section 3.11.2.1).

### 3.11.3 Hybridization

#### 3.11.3.1 Use of biotin-labeled DNA samples

#### Material:

Formamide (Fluka Co., Buchs, Switzerland), stored at -20°C

Denhardt's solution, concentrated 50 times: 1% Ficoll, 1% polyvinyl pyrrolidone, 1% BSA (w/v) in distilled water, stored at -20°C

Pipes, 0.5 M in distilled water, sterile-filtered

Herring sperm DNA (Mack Co., Illertissen): 10 mg/ml, sheared

DNA Detection System (BRL Co., Eggenstein)

Hybridization solution (final concentrations):

5x SSC

5x Denhardt's solution

Pipes 50 mM

Formamide 50% (v/v)

Herring sperm DNA 5% (v/v)

Following floating of the nitrocellulose filters or nylon membranes in 5xSSC and transfer into a plastic bag, 8 ml of hybridization solution were added for each 100 cm<sup>2</sup> of filter surface, for pre-hybridization, and then incubated for 1-3 hours at 42°C. Following removal of the solution, the biotin-labeled DNA sample, which was denatured for 10 min. at 95°C, was dissolved in 6 ml of hybridization solution per 100 cm<sup>2</sup> and transferred to the plastic bag. The filters were incubated overnight at 42-45°C. The filters were subsequently washed for 2 times 3 min. in 5x SSC/0.1 % SDS, 2 times 3 min. in 2x SSC/0.1% SDS, and, at 50°C, 2 times 15 min. in 0.3x SSC/0.1 % SDS.

The detection of the hybrids was accomplished by binding streptavidine to the biotinylated DNA probe. Through apposition with biotin labeled with alkaline phosphatase and the use of a substrate solution, it was possible to trigger an enzyme-catalyzed color precipitation. The reagents and buffer solutions were used in accordance with the manufacturers' instructions.

#### 3.11.3.2 Use of digoxigenin-labeled DNA samples

Material:

DNA Labeling and Detection Kit Nonradioactive (Boehringer Co., Mannheim)

Hybridization solution (final concentrations):

5x SSC

Blocking reagent, 5% (w/v)

Formamide, 50% (v/v)

N-lauroylsarcosine Na salt (Serva Co., Heidelberg) 0.1% (w/v)

SDS, 0.02% (w/v)